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Vacuolar Contents of Fruit Subepidermal Cells from *Vitis* Species[1]

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ABSTRACT

Enzymic treatment of mature, anthocyanin-containing grape berry subepidermal tissues from DeChaunac grapes released intact protoplasts. Filtration of the protoplast suspension through glass wool under mild suction resulted in the release of vacuoles. The total and individual contents of anthocyanins, flavonol glycosides, hydroxycinnamic acid esters, sugars, organic acids, and cations were determined in both tissue and vacuole preparations. A method for pH determination in intact vacuoles is reported. Based on the qualitative and quantitative anthocyanin composition, the average pH of the vacuoles was determined to be 2.7 (SD ± 0.17). The data suggest that anthocyanins are present in fruit subepidermal tissues in a noncomplexed form.

Anthocyanins are known to account for most of the orange, cerise, crimson, red, mauve, purple and blue colors occurring in the plant kingdom. The color differences among plants have been attributed to the nature of the pigment aglyca, the extent of glycosylation, the concentration of anthocyanin(s), complex formation with other flavonoid glycosides, metal complexing, and the pH of the cell sap (23). In flower petals, the anthocyanins are known to accumulate in the vacuoles of epidermal cells. In most fruits, they seem to be located in the vacuoles of a shallow subepidermal cell layer.

In this paper, we report the isolation of anthocyanin-containing vacuoles of grape berry subepidermal tissue and their qualitative and quantitative composition of anthocyanins and other compounds that are known to effect anthocyanin color, *e.g.* flavonol glycosides, hydroxycinnamic acid esters, sugars, organic acids, and cations. We also report the pH of the vacuolar sap, determined in intact vacuoles and in the subepidermal tissue, and show proof that complexing by metals or phenolic compounds plays no role in the color expression of mature grapes.

MATERIALS AND METHODS

Plant Material. DeChaunac grapes were grown in the vineyard of the New York State Agricultural Experiment Station, Geneva, NY, and harvested on September 6, 1978. The grapes were stored at 4 C until used. Additional grapes were harvested on August 28, 1979. Development of full color was used as a criterion for maturity.

Isolation of Grape Skins. For qualitative and quantitative determination of phenolic compounds, sugars, acids, and cations, the skins were removed manually from the grapes, rinsed briefly with distilled H_2O, frozen in N_2, and freeze-dried. To prevent oxidation of phenolic compounds, vacuum was released with N_2, and samples were stored in N_2 atmosphere.

Preparation of Vacuoles. Grape berries (1 kg) were rinsed briefly in 70% aqueous ethanol and dried on paper towels, and the skins were manually removed by gentle squeezing and cut in half. Batches of about 30 g of skins were rinsed with 100 ml buffer (17 mM citric acid, 70 mM Na_2HPO_4, and 1.2 M sorbitol at pH 6.0) for 30 min in 1,000-ml beakers on a reciprocal shaker (100 rpm). The buffer was replaced with a Cellulysin (1.5%; Calbiochem) and Macerase (1%; Calbiochem) solution in the same volume as the above buffer. The grape skins were digested for 7 h at room temperature, the incubation medium decanted, and the protoplasts sedimented at 100*g* in a centrifuge. The sediment was resuspended in the rinsing buffer from above (1,500 ml) and filtered through a glass wool layer in a Büchner funnel under mild suction (aspirator). This treatment produced vacuoles from protoplasts. The vacuoles were sedimented at 100*g*, resuspended in 225 ml rinsing buffer, and sedimented by gravity twice. The number of vacuoles in a preparation was determined in a well slide using 5 μl aliquot and 20 μl buffer.

Disruption of Vacuoles. The vacuole preparations were transferred into 40-ml centrifuge tubes and the vacuoles sedimented at 50*g*. The supernatant buffer was decanted, the volume made up to 40 ml with distilled H_2O, and the preparation homogenized by sonication. Microscopic examination of this preparation showed that all vacuoles had lysed. The homogenate was centrifuged at 180,000*g* for 2 h to remove membranous material. The supernatant (37 ml) was decanted, frozen in N_2, and stored at −40 C.

Identification of Anthocyanins. The anthocyanins in DeChaunac grapes have been previously identified (28).

Determination of Total Anthocyanin Content in Grape Skins. Freeze-dried grape skin powder (1.0 g) was extracted with 0.1% aqueous HCl (100 ml), the absorbance of this solution measured at 520 nm, and the anthocyanin concentration determined using $\epsilon = 33{,}000\ l \cdot mol^{-1} \cdot cm^{-1}$ (27).

Determination of the Total Anthocyanin Content in Vacuole Preparations. One ml of the vacuole lysate was used for the determination, as described above.

Quantitative Determination of the Individual Anthocyanins. The individual anthocyanin content of grape skins and vacuole preparations was determined by quantitative adaptation of a previously described HPLC method (28). Only pigments present in significant amounts were quantitated. For the establishment of anthocyanin standard curves, the following pigment stock solutions were prepared: delphinidin 3,5-diglucoside, 28.5 μM; cyanidin 3,5-diglucoside, 40.5 μM; petunidin 3,5-diglucoside, 72.5 μM; malvidin 3,5-diglucoside, 119 μM; delphinidin 3-glucoside, 78.0 μM, petunidin 3-glucoside, 204 μM; and malvidin 3-glucoside, 47.0 μM dissolved in 1.0 ml distilled H_2O. Aliquots (0–25 μl) of the original or diluted

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stock solutions were injected and the standard curves determined by measuring peak height of the pigment samples. All anthocyanin preparations were from our laboratory collection.

Vacuole lysates and grape skin extracts (10 μl aliquots) were injected directly in the chromatograph, and grape skin extracts were prepared as follows: one g freeze-dried grape skin powder was extracted with 2 × 75 ml 5% aqueous acetic acid at 4 C for 30 min, centrifuged at 15,000*g*, concentrated to about 3 ml, and diluted to 30 ml with distilled H_2O. The solution was then filtered, and an aliquot (10 μl) was chromatographed.

Isolation and Identification of Flavonol Glycosides. Freeze-dried grape skin powder (500 g) was extracted with 2 × 1,500 ml hot methanol and with 1,000 ml hot 80% aqueous methanol. The insoluble residue was removed by centrifugation, and the supernatant was filtered and concentrated under reduced pressure at 40 C to 1.5 liters. The concentrate was extracted with 3 × 200 ml hexane to remove lipids, Chl, and carotenoids. The organic solvents were removed and 300 ml H_2O added, and the aqeuous residue (about 1 liter) was centrifuged at 27,000*g*. The supernatant was percolated through a polyamide column (4.5 × 100 cm), the column washed with 2 liters water to remove sugars and organic acids, and the anthocyanins removed by washing with 4 liters 50% aqueous methanol. The column was then eluted with a linear methanolic gradient (4 liters, 50% aqueous methanol → 100% methanol) and washed further with 4 liters methanol. The elution was monitored by TLC in 15% aqueous acetic acid. Four fractions (1–4) were obtained. Further elution of the column with a linear acidity gradient (methanol → methanol −0.004 N HCl, 4 liters) and continuous washing with an additional 4 liters acidic methanol removed five additional fractions (fractions 5–9).

Fractions 1 to 4 and 9 contained flavonol glycosides. The individual flavonol glycosides were further purified from the above five fractions by rechromatography on polyamide columns and by preparative TLC. Five flavonol glycosides were isolated: quercetin 3-rutinoside (50 mg); quercetin 3-glucoside (12 mg); myricetin 3-glucoside (36 mg); quercetin 3-glucuronide (13 mg); and quercetin 3-methylglucuronide (67 mg). These compounds were identified by UV-VIS spectral analysis, cochromatography with authentic reference compounds in six solvent systems (8, 11), and analysis of their acid hydrolysis products.

Quantitative Determination of the Individual Flavonol Glycosides. The individual flavonol glycoside content of the grape skin extract and vacuole lysate was determined by HPLC on a ZORBAX ODS column (4.6 × 25 mm; DuPont Company) with a minor modification of the method described by Asen (2, 3). Ten-μl samples were analyzed with a linear gradient (20 → 100% tetrahydrofuran in H_2O) with a column flow rate of 1.4 ml/min.

Flavonol glycoside standard curves for the quantitative determination were established with the following stock solutions in 1.0-ml volumes: quercetin 3-rutinoside (1.14 mM); quercetin 3-glucoside (1.36 mM); myricetin 3-glucoside (0.74 mM); and quercetin 3-glucuronide (1.50 mM). Quantitation was based on peak height at 525 nm.

Preparation of Grape Skin and Vacuole Lysate Extracts. Freeze-dried grape skin powder (1.0 g) was extracted with 3 × 50 ml hot 70% aqueous methanol. The extract was centrifuged and the supernatant concentrated until all methanol was removed. The aqueous concentrate was extracted with hexane, and the residual hexane was removed in vacuum at 40 C. The flavonol glycosides were extracted from the aqueous concentrate with 4 × 10 ml ethyl acetate. The ethyl acetate extract was evaporated to dryness, and the residue was dissolved in 1.0 ml methanol and filtered through a 0.2-μm filter. Ten-μl aliquots of this solution were used for the quantitative analysis.

Vacuole preparations were extracted with 3 × 50 ml ethyl acetate and processed as above.

Isolation and Identification of Hydroxycinnamic Acid Esters. Fractions 5 to 8 from the polyamide column separation from above were rechromatographed on polyamide (4 × 25 cm) with the linear acidity gradient to yield two major fractions. *p*-Coumaryltartrate and caffeyltartrate were further purified by preparative TLC and Sephadex LH-20 chromatography. Both compounds (*p*-coumaryltartrate, 10 mg; caffeyltartrate, 5 mg) were identified by comparison of their chromatographic (in 6 solvents), UV- VIS-, MS- and NMR-spectral properties with authentic reference compounds (gift from Dr. V. L. Singleton).

Quantitative Determination of Hydroxycinnamic Acid Esters. The quantitative determination of the hydroxycinnamic acid esters was carried out on HPLC, according to the method described by Ong and Nagel (19). Ten-μl samples were injected onto ZORBAX ODS columns (4.5 × 250 mm), eluted with a 5% aqueous acetonitrile solution (pH 2.6, adjusted with H_3PO_4) for 15 min, and followed by a linear gradient from 6 to 16% aqeuous acetonitrile over a period of 15 min, with a solvent flow rate of 2.5 ml/min. Standard curves for *p*-coumaryl- and caffeyltartrate were prepared from methanolic stock solutions (2×10^{-5} to 4.5×10^{-4} M/l in both cases). Vacuole lysates were analyzed directly. Grape skin extracts were prepared as follows: one g grape skin powder was extracted with hot 80% aqueous methanol, the methanol was evaporated, and the aqeuous residue was extracted with hexane to remove Chl and carotenoids. The aqueous residue was filtered (0.2-μm filter), diluted to 20 ml with methanol, and analyzed (10 μl).

Identification and Quantitative Determination of Carbohydrates. Sugars were identified by HPLC, according to the method of Conrad and Palmer (9), on a μ-Bondapak carbohydrate analysis column (4.5 × 250 mm) using a refractive index detector, with 85% aqueous acetonitrile as solvent. The flow rate was 2.0 ml/min. Five-μl samples were used from stock solutions of glucose, fructose, and sucrose to establish the standard curves. Grape skin samples were prepared as follows: 2 g freeze-dried grape skin powder were extracted with hot 80% aqueous ethanol for 30 min, and the extract was centrifuged and evaporated to about 5 ml. This concentrate was percolated through a PVP column (12.5 × 100 mm) and washed with H_2O. The eluate was percolated through a Dowex 50 w × 8-H^+ column (15 × 100 mm), and the column was washed with H_2O. The effluent was evaporated to dryness, and the residue was taken up in 1.7 ml H_2O. This solution was used for HPLC analysis.

Vacuole preparations were concentrated to 5 ml and treated as above, and the final residue was dissolved in 5.0 ml H_2O and analyzed.

Identification and Quantitative Determination of Anions. One g freeze-dried grape skin powder was extracted with 3 × 20 ml boiling H_2O. The extract was centrifuged and the supernatant made up to 100 ml. The acids were precipitated as lead salts (17) from 20 ml of the above solution, trimethylsilylated, and analyzed (10 μl) by GLC.

Standard solutions of citric, malic, tartaric, quinic, and phosphoric acids for the quantitative analysis were prepared similarly, and standard curves were established with 2 μl of the preparation, with undecanoic acid in pyridine (4 mg/ml) as internal standard. The peak area in the gas chromatograms was used for the quantitative determination. Vacuole lysates (10-ml aliquots) were treated similarly.

Identification and Quantitative Determination of Cations. One g freeze-dried grape skin was boiled with concentrated HNO_3 (50 ml) until about 10 ml of the suspension remained. This was diluted to 50 ml with distilled H_2O and filtered. A blank was prepared similarly. Vacuole lysates were used directly (Ca^{2+}, Mg^{2+}) or evaporated to dryness and digested as above. This final solution was used for cation determination by atomic absorption spectroscopy. For the quantitative determination, standard curves were established with Ca^{2+}, Mg^{2+}, Al^{3+}, $Fe^{2+,3+}$, Mn^{2+}, Cu^{2+}, K^+, and

Na^+. The analyses were carried out with a Perkin Elmer 305B AA spectrometer (Perkin Elmer, Mountainview, CA), according to the Perkin Elmer Reference Manual (March, 1971).

Determination of the Vacuolar pH. The vacuolar pH was determined spectrophotometrically based on the anthocyanin content. Anthocyanins in the pH range of 1 to 4 exist in two different structural transformation forms: the red-colored flavylium cation [*F*] and the colorless carbinol base [*C*] (12). The equilibrium concentration of these two structural transformation forms is pH-dependent. Therefore, the total anthocyanin content [*A*] can be expressed as follows:

$$[A] = [F] + [C] \quad (1)$$

The carbinol base concentration [*C*] is calculated by subtracting [*F*] from the total anthocyanin concentration [*A*], established in model solutions or calculated in vacuole or skin preparations:

$$[A] = \frac{A_{515\ nm}}{\epsilon \cdot l}\ \text{M/l}$$

ϵ = 33,000 l/M·cm (27) is the average molar absorption coefficient for anthocyanins; l = path length of light in anthocyanin containing tissue or vacuole preparation.

The equilibrium between the red flavylium cation [*F*] and the colorless carbinol base [*C*] concentration can be represented by the Henderson-Hasselbach equation:

$$\text{pH} = -B \log ([F]/[C]) + pK_a \quad (2)$$

In this equation, B is a constant (under normal circumstances the value is 1) and represents the slope of the curve. By plotting pH *versus* log ([*F*]/[*C*]), as shown in Figure 1 with a model anthocyanin solution representing the composition and concentration of the pigments in the vauole in 0.15 M citrate-phosphate buffer, this value is calculated to be −1.48. B deviates from 1 because of self association (5) of anthocyanins at higher concentrations. The x-intercept, pK_a, is determined to be 2.59. Therefore, equation (2) yields:

$$\text{pH} = -1.48 \log ([F]/[C]) + 2.59 \quad (3)$$

Rearranging equation (2) for the [*F*]/[*C*] ratio yields:

$$\log ([F]/[C]) = 1.75 - 0.67\ \text{pH} \quad (4)$$

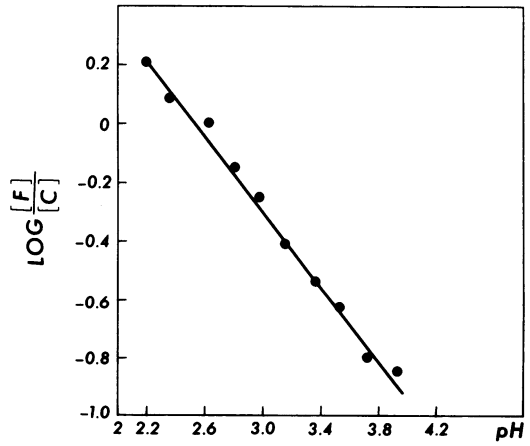

FIG. 1. Calibration curve for determination of the [*F*]/[*C*] ratios as function of (pH). The total anthocyanin content [*A*], determined in 0.1% HCl extract from vacuole or grape skin preparations, is expressed by the equation [*A*] = [*F*] + [*C*]. The carbinol base concentration [*C*] is calculated by subtracting the concentration of the flavylium ion [*F*] from the total anthocyanin content [*A*]. [*A*] is determined as described in "Materials and Methods."

Substituting the ratio [*F*]/[*C*], measured and calculated from intact grape skins and/or vacuole preparations in equation (3), gives the true vacuolar pH value.

Measurement of [*F*]/[*C*] in Intact Grape Skins. Freshly removed grape skins were positioned between the two faces of quartz micro cells, and the A was measured at 515 nm against a similarly treated, nonpigmented section of another grape skin as a blank. Each skin was then homogenized in 0.5 N HCl in an Eppendorf micro test tube (Brinkmann Institute, Westbury, NY) and centrifuged at 12,000*g*; the supernatant was decanted and diluted to 100 ml with the same solvent, and the absorbance was measured. [*F*] was determined by calculating the volume of the grape skin (area × 100 μm, the average thickness of the pigmented vacuole layers in the tissue, as measured in the microscope); [*A*] was determined from the anthocyanin extract. [*C*] was calculated from equation (1).

[*F*]/[*C*] in vacuole preparations was determined similarly, as described above, with an aliquot of the preparation in micro quartz cells with 100-μm path length and after disruption of the vacuole preparation in 0.5 N HCl.

RESULTS

Vacuole Preparations. Incubation of the grape skin pigments in the Cellulysin/Macerase-containing buffer for 7 h resulted in the production of large numbers of partially or fully intact protoplasts, as shown in Figure 2. Filtration of the protoplast suspension through glass wool produced vacuoles. Two hundred mature grape skins produced about 1.2 to 1.9 × 10^7 vacuoles. These vacuoles retained some fragments of the particulate cytoplasm and/or plasma membrane (Fig. 3), which could not be removed by further purification. The yield of vacuoles from the skin tissues was between 2 and 5.4%, as determined by their anthocyanin content. The isolated vacuoles varied in both color intensity and size. The average diameter of the vacuoles was measured in a hemocytometer to be 32 μm. The average volume of a vacuole was, therefore, calculated to be 17 picoliters. Since the diameter of the vacuoles varied from 5 to 80 μm, extreme vacuolar volumes were calculated to be 0.065 picoliter and 268 picoliters, respectively. Ten vacuole preparations were made. The number of vacuoles and their total anthocyanin content is shown in Table I.

Anthocyanin Composition and Content of Vacuoles. The anthocyanin composition of DeChaunac grapes has been previously

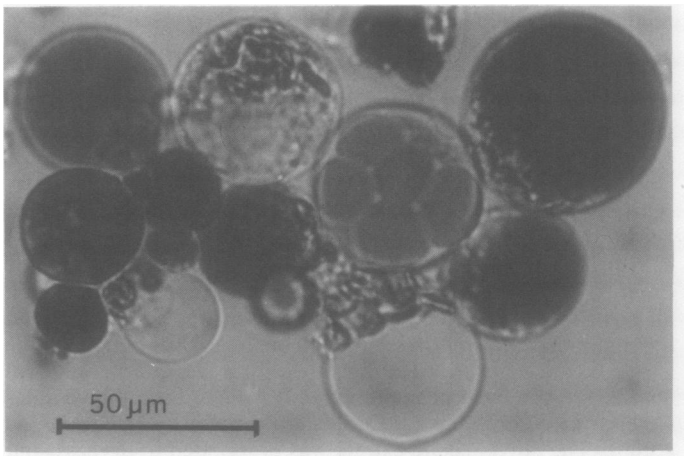

FIG. 2. Protoplast preparation from mature DeChaunac grape berry subepidermal tissues. Protoplasts were prepared by incubation of about 30-g skins with 1.5% Cellulysin and 1% Macerase in 100 ml buffer containing 17 mM citric acid, 70 mM Na_2HPO_4, and 1.2 M sorbitol (pH 6.0) for 7 h at room temperature. The protoplasts were sedimented at 100*g* in a centrifuge.

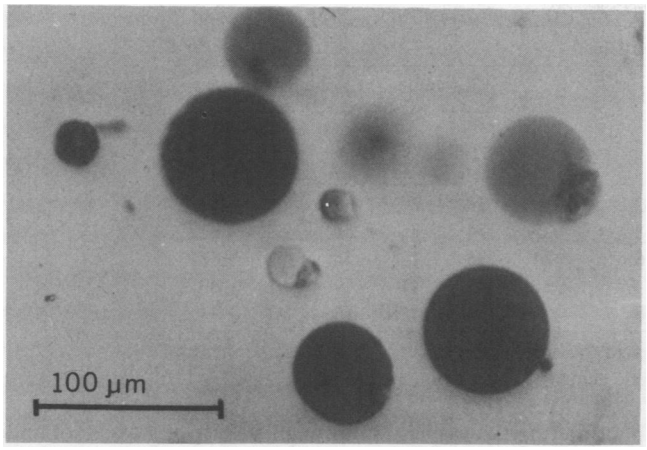

FIG. 3. Vacuole preparation from mature DeChaunac grape berry subepidermal tissues. The sedimented protoplasts from Figure 2 were resuspended in 1,500 ml incubation buffer not containing Cellulysin and Macerase and filtered through a layer of glass wool under mild suction with an aspirator. The vacuoles were sedimented at 100*g*, resuspended in the above buffer, and sedimented by gravity twice.

Table I. *Yield and Total Anthocyanin Content of Vacuole Preparations from Mature Vitis cv. DeChaunac Grape Skins*

Vacuole Preparation	No. of Vacuoles	Anthocyanin Concentration		
		Total mol in preparation	pmol per Vacuole	Vacuolar concentration
				M
1	12.0×10^6	2.53×10^{-5}	2.11	0.12
2	18.9×10^6	3.77×10^{-5}	1.99	0.12
3	19.2×10^6	3.64×10^{-5}	1.90	0.11
4	15.2×10^6	3.80×10^{-5}	2.50	0.15
5	15.5×10^6	2.24×10^{-5}	1.45	0.085
6	16.0×10^6	2.56×10^{-5}	1.60	0.094
7	18.5×10^6	2.47×10^{-5}	1.33	0.078
8	18.5×10^6	2.47×10^{-5}	1.33	0.078
9	11.9×10^6	1.35×10^{-5}	1.13	0.066
10	11.9×10^6	1.35×10^{-5}	1.13	0.066
Avg	15.76×10^6	2.62×10^{-5}	1.647	0.0967
SD	$\pm 3.00 \times 10^6$	$\pm 8.93 \times 10^{-6}$	±0.459	±0.0276
SE	$\pm 9.94 \times 10^5$	$\pm 2.82 \times 10^{-6}$	±0.145	±0.00871
C[a]	19.05%	34.05%	27.9%	28.49%

[a] Coefficient of variation.

identified (28). HPLC investigation of the freeze dried grape skins showed that the major anthocyanin components were the 3,5-diglucosides of cyanidin, delphinidin, petunidin, and malvidin, and the 3-glucosides of petunidin and malvidin. The total anthocyanin content of fresh grape skin was determined to be 2.88 µmol/g.

Calculated from data presented in Table I, the average anthocyanin content of vacuoles was 1.65 pmol, resulting in a vacuolar concentration of 97 mM. The sum of the individually determined anthocyanins fits into the same population with similar statistical parameters.

Flavonol Glycoside Composition and Content of Vacuoles. Five flavonol glycosides were identified in DeChaunac grape skin. These are the 3-glucoside, 3-rutinoside, 3-glucuronide, and 3-glucuronide methyl ester of quercetin and myricetin 3-glucoside (Table II).

HPLC examination of the freeze-dried grape skin powder, and that of the vacuole lysate, showed only the presence of four flavonol glycosides, quercetin 3-glucoside, 3-rutinoside, 3-glucuronide, and myricetin 3-glucoside (Fig. 4). Quercetin 3-glucuronide methyl ester was not present in the chromatogram. The formation of this compound is apparently due to an artifact during column chromatographic isolation using methanolic HCl as solvent (1, 5, 15). Fresh grape skin contained quercetin 3-glucuronide in highest amounts, 0.099 µmol/g, while the compound present in highest concentration in the vacuole lysate was quercetin 3-glucoside (Table III).

Hydroxycinnamic Acid Ester Composition and Content of Vacuoles. HPLC investigation of both grape skins and vacuole preparations showed the presence of two major and two minor hydroxycinnamic acid esters (Fig. 5). The major compounds were identified by UV-VIS, MS, and NMR spectroscopy and by their chromatographic properties as the tartrate esters of *p*-coumaric and caffeic acids. Their concentration in fresh grape skins was found to be 0.222 and 0.757 $\mu mol \cdot g^{-1}$, respectively. Vacuolar concentration of *p*-coumaryl tartrate was determined to be 4.05×10^{-3} M, and that of caffeyl tartrate was 1.65×10^{-2} M (Table III).

Carbohydrate, Anion and Cation Content of Vacuoles. HPLC examination of both fresh grape skin and vacuole preparations showed the presence of three sugars in significant amounts. These were identified as glucose, fructose, and sucrose. While both glucose and fructose were present in nearly identical amounts (about 1.5 M), the sucrose concentration in both grape skins and vacuoles was less than 10% of the other two sugars (Table IV).

Tartaric, malic, quinic, phosphoric, and citric acids were found to be the major anions in both grape skins and vacuole lysates. These compounds were present at 0.2 to 1.10 M concentration in the vacuoles (Table IV).

Analysis of grape skins and vacuole lysates for cations by atomic absorption spectrometry showed the presence of K^+, Ca^{2+}, Mg^{2+}, $Fe^{2+,3+}$, Na^+, Al^{3+}, Cu^{2+}, and Mn^{2+} in significant amounts. Potassium was present at 2.73 M concentration in the vacuoles, while the concentration of other cations was in the mM range (Table IV). The concentration of Na could not be determined in vacuole lysates because of the buffer system used in the isolation.

Determination of the Vacuolar pH. The pH of the vacuolar sap was determined in all 10 vacuole preparations with the spectrophotometric method. The average pH was found to be 2.78, with extreme values measured at 2.54 and 2.99 for individual vacuole preparations (Table V). Direct measurement of pH in grape skin macerates and extracts resulted in significantly higher values of 4.06 and 3.99, respectively.

DISCUSSION

DeChaunac grape is a complex *Vitis* interspecies hybrid containing 80 to 85% anthocyanidin 3,5-diglucosides in its pigment composition. Anthocyanidin 3-glucosides are present at the 10 to 15% level; acylated anthocyanins occur only in trace amounts. Microscopic examination of a cross section of mature berries showed that, in this *Vitis* cultivar, the anthocyanins are localized in a shallow subepidermal layer. Contrary to petal and leaf tissues, where anthocyanins have been observed to accumulate in the epidermal layers (13, 25), the epidermis of DeChaunac grape berries is devoid of anthocyanins. Flavone and flavonol glycosides, especially if present in small concentrations, can not be detected visually in tissues. Since their biosynthetic path differs most likely only by four enzymes from that of the anthocyanins, they are assumed to be absent from the epidermis also. The first subepidermal layer seems to contain uniformly dark red colored vacuoles. The following two cell layers contain significantly smaller amounts of the pigments. Anthocyanins were observed to occur sporadically in the 4th, 5th, and 6th cell layers. The occurrence of anthocyanin-containing vacuoles was not observed in deeper lying

Table II. *UV-VIS Spectral and Chromatographic Properties of the Major Flavonol Glycosides Isolated from DeChaunac Grape Skins*

Compound	Spectral Properties (λ_{max} nm [ϵ] in Methanol)	R_F values for Solvent[a]					
		1	2	3	4	5	6
Quercetin 3-glucoside	360 [21,200], 262	8	33	28	68	66	67
Quercetin 3-rutinoside	364 [19,500], 258	25	54	25	58	56	82
Quercetin 3-glucuronide	359 [17,000], 256	74	35	15	55	62	72
Quercetin 3-glucuronide methyl ester	360, 258	11	45	41	79	81	80
Myricetin 3-glucoside	363 [19,100], 258	5	24	13	50	48	74

[a] Chromotographic solvents: 1, H_2O; 2, 15% aqueous acetic acid; 3, benzene:acetic acid:H_2O (6:7:0.9); 4, phenol:H_2O (73:27); 5, butanol:acetic acid:H_2O (6:1:2); 6, butanol:acetic acid:H_2O (4:1:5), organic phase.

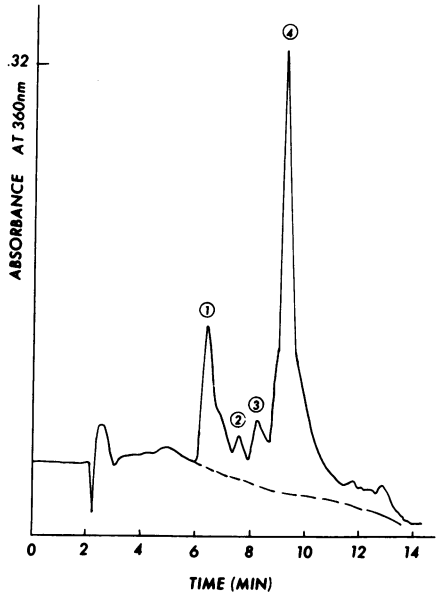

FIG. 4. HPLC determination of flavonol glycosides in DeChaunac grape subepidermal tissues on a ZORBAX ODS column (4.6 × 25 mm). Solvent, linear aqueous tetrahydrofuran gradient (20 to 100%); flow rate, 1.4 ml/min. Identity of peak 1, quercetin 3-rutinoside; peak 2, myricetin 3-glucoside; peak 3, quercetin 3-glucoside; peak 4, quercetin 3-glucuronide. (- - -), Base line of chromatogram.

cells in this grape cultivar.

The removable skin of the berry contains all the anthocyanin-containing cells. Therefore, fresh and freeze-dried skins were used in the preliminary experiments to identify the individual anthocyanins, flavonol glycoside, hydroxycinnamic acid ester, sugar, anion, and cation components, and to quantitate them. The quantitative data obtained from the grape skins do not permit any conclusion on the above compounds' concentration in the vacuole. These are important, however, in the establishment of the relative concentration between groups of individual components.

Incubation of the grape skins with the Cellulysin-Macerase mixture produced protoplasts in acceptable yields within 7 h at 1.2 M sorbitol concentration in the incubation mixture. At lower sorbitol or mannitol concentrations, the protoplasts lysed within a short time. In this respect, the grape skin tissue closely resembles other, high sugar-containing, tissues such as beet roots, which also require high osmolarity medium for the production of stable protoplasts and vacuoles (14). The protoplasts produced from the grape skin did not release intact vacuoles upon treatment with 0.2 M $Na_4P_2O_7$-HCl (pH 8.0) (26), upon dilution of the osmoticum (20), by polybasic DEAE-dextran treatment (10), or by shearing forces created upon uptake and expulsion in a syringe (18). Vacuoles were produced in about 55% yield from protoplasts, upon filtering the suspension through a layer of glass wool under mild suction.

Because of the high sugar content of the vacuoles, sucrose density and Ficoll gradients could not be used in further purification of the vacuole preparations. Purification was accomplished by repeated filtration through glass wool in the rinsing buffer (17 mM citric acid, 70 mM Na_2HPO_3, and 1.2 M sorbitol at pH 6.0). The lability of the vacuoles at pH values higher than 7 is possibly due to the relatively high concentration of tartaric acid in vacuoles of the *Vitis* species. In this respect, the metabolism in grapes resembles that of geraniums, which are also known to accumulate higher concentrations of tartaric acid (29). Microscopic examination of the vacuole preparations showed that the majority contained particulate cytoplasmic fragments adhering as polar caps. Addition of BSA or EDTA, previously reported to remove cytoplasmic fragments from vacuoles successfully (14, 20), had no effect on grape skin vacuole preparations. Because the soluble cytoplasmic contents and the majority of the particulate cytoplasm were removed during vacuole release and the residual particulate cytoplasmic contents after lysis of the vacuoles by 180,000*g* centrifugation, it is considered that these have not seriously interfered with the determination of the vacuolar contents.

Total and individual anthocyanin contents of the vacuoles for the major pigments, ranging from 2.9 to 97 mM, approach their limits of solubility. At these concentrations, it is expected that self association of the pigments (5, 21, 24) plays a significant role in color expression. While the individual anthocyanin contents of vacuoles have been reported for the first time and there are no comparative data in the literature, the total anthocyanin concentration in vacuoles of DeChaunac grape berry subepidermal tissue is one order of magnitude higher than that reported for other plants (4, 25). The vacuolar concentration of hydroxycinnamic acid esters approaches that of the anthocyanins. Both caffeyl- and *p*-coumaryl tartrate were found to have no significant effect on pigment color at these concentrations. The concentration of flavonol glycosides is about two orders of magnitude lower than that of the anthocyanins. Inasmuch as significant effects on anthocyanin color with flavonol glycosides were found to take place at equimolar or higher flavonol glycoside ratios (21), their presence at 10^{-4} to 10^{-5} M quantities has no effect on anthocyanin color.

The major carbohydrate components of the vacuoles are glucose and fructose, both present at the same concentration (1.45 and 1.51 M), and comparatively small amounts of sucrose (0.1 M). These high sugar concentrations explain the lability of the protoplasts and vacuole preparations below 1.2 osmolarity levels. Comparative vacuolar carbohydrate concentrations in storage tissues are 1.5 M sucrose in castor bean endosperm (18) and substantial but not precisely determined amounts in red beet roots (16). Vacuoles isolated from petal tissues contain lesser amounts of carbohydrates: 29 mM glucose in *Hippeastrum* and 154 mM in *Tulipa* (25).

The major anions in grape skins were found to be tartaric,

Table III. *Anthocyanin, Flavonol Glycoside, and Hydroxycinnamic Acid Ester Composition of DeChaunac Grape Berry Subepidermal Tissue and Vacuoles*

	Concentration		
	Subepidermal tissue	Vacuoles	
	μmol·g⁻¹ fresh wt	*pmol/vacuole*	*M*
Anthocyanins			
Delphinidin 3,5-diglucoside	0.352	1.36×10^{-1}	8.00×10^{-3}
Cyanidin 3,5-diglucoside	0.225	8.15×10^{-2}	4.79×10^{-3}
Petunidin 3,5-diglucoside	0.733	3.81×10^{-1}	2.24×10^{-2}
Malvidin 3,5-diglucoside + cyanidin 3-glucoside	1.23	6.37×10^{-1}	3.75×10^{-2}
Petunidin 3-glucoside	0.254	4.29×10^{-2}	2.52×10^{-3}
Malvidin 3-glucoside	0.0898	1.64×10^{-2}	9.65×10^{-4}
Flavonol glycosides			
Quercetin 3-rhamno glucoside	0.0503	1.22×10^{-3}	7.18×10^{-5}
Myricetin 3-glucoside	0.0132	6.35×10^{-4}	3.73×10^{-5}
Quercetin 3-glucoside	0.0240	4.39×10^{-3}	2.58×10^{-4}
Quercetin 3-glucuronide	0.0994	1.80×10^{-3}	1.06×10^{-4}
Hydroxycinnamic acid esters			
p-Coumaryl tartrate	0.222	6.88×10^{-2}	4.05×10^{-3}
Caffeyl tartrate	0.757	2.81×10^{-1}	1.65×10^{-2}

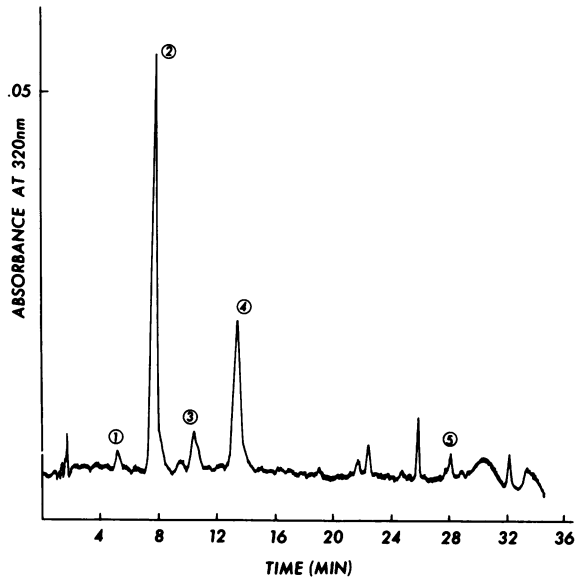

FIG. 5. HPLC Separation of hydroxycinnamic acid esters in DeChaunac grape subepidermal tissues on ZORBAX ODS column (4.5 × 250 mm). After injection of the sample (10 μl), the column was eluted initially for 15 min with a 5% aqueous acetonitrile solution (pH 2.6, adjusted with H_3PO_4) and thereafter with a linear aqueous acetonitrile gradient (6 to 16%) for additional 15 min. Flow rate, 2.5 ml/min. Identity of peak 1, unknown; peak 2, caffeyl tartrate; peak 3, unknown; peak 4, *p*-coumaryltartrate; peak 5, *p*-coumaric acid. Other peaks in the chromatogram are caused by interference from flavonol glycosides and anthocyanins.

Table IV. *Carbohydrate, Anion, and Cation Composition of DeChaunac Grape Berry Subepidermal Tissues and Vacuoles*

	Concentration		
	Subepidermal tissue	Vacuoles	
	μmol·g⁻¹ fresh wt	*pmol/vacuole*	*M*
Carbohydrates			
Glucose	136	24.7	1.45
Fructose	140	25.6	1.51
Sucrose	9.36	1.71	0.10
Anions			
Phosphoric	1.33	ND[a]	ND
Malic	17.0	5.31	0.31
Tartaric	26.7	18.6	1.09
Citric	0.83	ND	ND
Quinic	2.19	3.39	0.20
Cations			
K^+	88.2	46.3	2.73
Ca^{2+}	3.94	1.40×10^{-1}	8.24×10^{-3}
Mg^{2+}	3.37	8.90×10^{-1}	5.23×10^{-2}
$Fe^{2+,3+}$	0.0645	2.00×10^{-2}	1.18×10^{-3}
Al^{3+}	0.115	5.89×10^{-3}	3.46×10^{-4}
Cu^{2+}	0.0245	5.08×10^{-3}	2.99×10^{-4}
Mn^{2+}	0.0391	1.88×10^{-3}	1.11×10^{-4}
Na^+	1.35	ND	ND

[a] ND, Not determined.

malic, phosphoric, quinic, and citric acids. Phosphoric and citric acids could not be determined in vacuolar lysates because they were components of the isolating buffer. Tartaric acid is the main anionic vacuolar constituent (1.09 M), followed by malic (0.31 M) and quinic (0.20 M) acids. This high concentration of tartaric acid may explain the relatively low pH of the vacuolar sap.

The pH values obtained by direct measurement of grape skin macerates or extracts were approximately 1 unit higher than were those determined by the spectrophotometric method. This is most likely due to mixing of basic cytoplasmic components with vacuolar contents.

Recombination of vacuolar anionic components on a quantitative basis resulted in a solution with the pH value of 2.3. This pH is rather acidic and is arrived at by not considering the presence of cations.

Investigations of the cation composition and content of the vacuole lysate showed that potassium was the major component present at a 2.73 M concentration in the vacuoles. Other cations

Table V. *Spectrophotometric Determination of the Vacuolar pH in Intact Vacuoles Prepared from DeChaunac Grape Berry Subepidermal Tissues*

Vacuole Preparation No.	A_{515} nm of the Preparation	$[F]$	$A \cdot cm^{-1}$ Acidic Extract	$[A]$	$[C]$	$\log\frac{[F]}{[C]}$	Vacuolar pH
		M		M			
1	1.76	5.34×10^{-3}	0.375	11.36×10^{-3}	6.02×10^{-3}	−0.052	2.67
2	3.31	10.04×10^{-3}	0.890	27.00×10^{-3}	16.96×10^{-3}	−0.228	2.93
3	3.61	10.94×10^{-3}	0.710	21.52×10^{-3}	10.58×10^{-3}	0.0145	2.57
4	2.54	7.70×10^{-3}	0.670	20.32×10^{-3}	12.62×10^{-3}	−0.215	2.91
5	3.63	11.00×10^{-3}	0.485	14.70×10^{-3}	3.70×10^{-3}	0.473	
6	3.16	9.58×10^{-3}	0.800	18.18×10^{-3}	8.60×10^{-3}	0.045	2.54
7	3.19	9.66×10^{-3}	0.690	20.92×10^{-3}	11.26×10^{-3}	−0.066	2.69
8	3.32	10.06×10^{-3}	0.950	28.80×10^{-3}	18.74×10^{-3}	−0.270	2.99
9	3.90	11.82×10^{-3}	1.01	30.60×10^{-3}	18.78×10^{-3}	−0.201	2.89
10	3.80	11.52×10^{-3}	0.920	27.88×10^{-3}	16.36×10^{-3}	−0.152	2.81

(Mg^{2+}, Ca^{2+}, $Fe^{2+,3+}$, Al^{3+}, Cu^{2+}, Mn^{2+}) were present at concentrations two to four orders of magnitude lower, and, hence, cannot be considered to play an important role in the establishment of the pH value.

The pH of the vacuolar sap was determined to be 2.78 with the method described above. While this method is tedious and requires the preliminary determination of the anthocyanin composition and content of the vacuoles, it is presently the closest approximation of the true vacuolar pH in living tissues.

Optical measurements, not considering the self association effect of anthocyanins at high concentrations (which result in a hypsochromic shift of the λ_{max} of the pigment solutions and a quasiexponential increase in their absorbance [7, 21]), are liable to give results deviating grossly from the true value.

The pH of 2.78 is an average value obtained from measurements from 10 individual vacuole preparations. Extreme values measured were pH 2.54 and pH 2.93, apparently caused mainly by the presence of tartaric acid at high concentrations. Supporting evidence for such a low pH value within intact vacuoles is obtained by microspectrophotometric measurements on young geranium petals. Geranium petal vacuoles also accumulate tartaric acid in large quantities (29), and their vacuolar pH has been determined to be 2.9 (22).

Color expression in flowers, where the pH of the vacuolar sap is generally around 4 to 6, has been attributed to anthocyanin-flavonol glycoside complexes (6), complexing with hydroxycinnamic acid residues and/or metals (23). The same factors were assumed to control the color of fruits also. The above data suggest that, in fruit subepidermal tissues where anthocyanins are accumulated, complex formation with other flavonoid compounds, hydroxycinnamic acid derivatives, and metals is not involved in the color expression phenomena. The color of the investigated grape is caused by anthocyanins alone, present in the vacuoles of three to six subepidermal cell layers at high concentrations in a very acidic environment. Self association effects are most likely involved in color expression, as is light scattering and the high density (for light penetration) of the underlying pericarp tissue.

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